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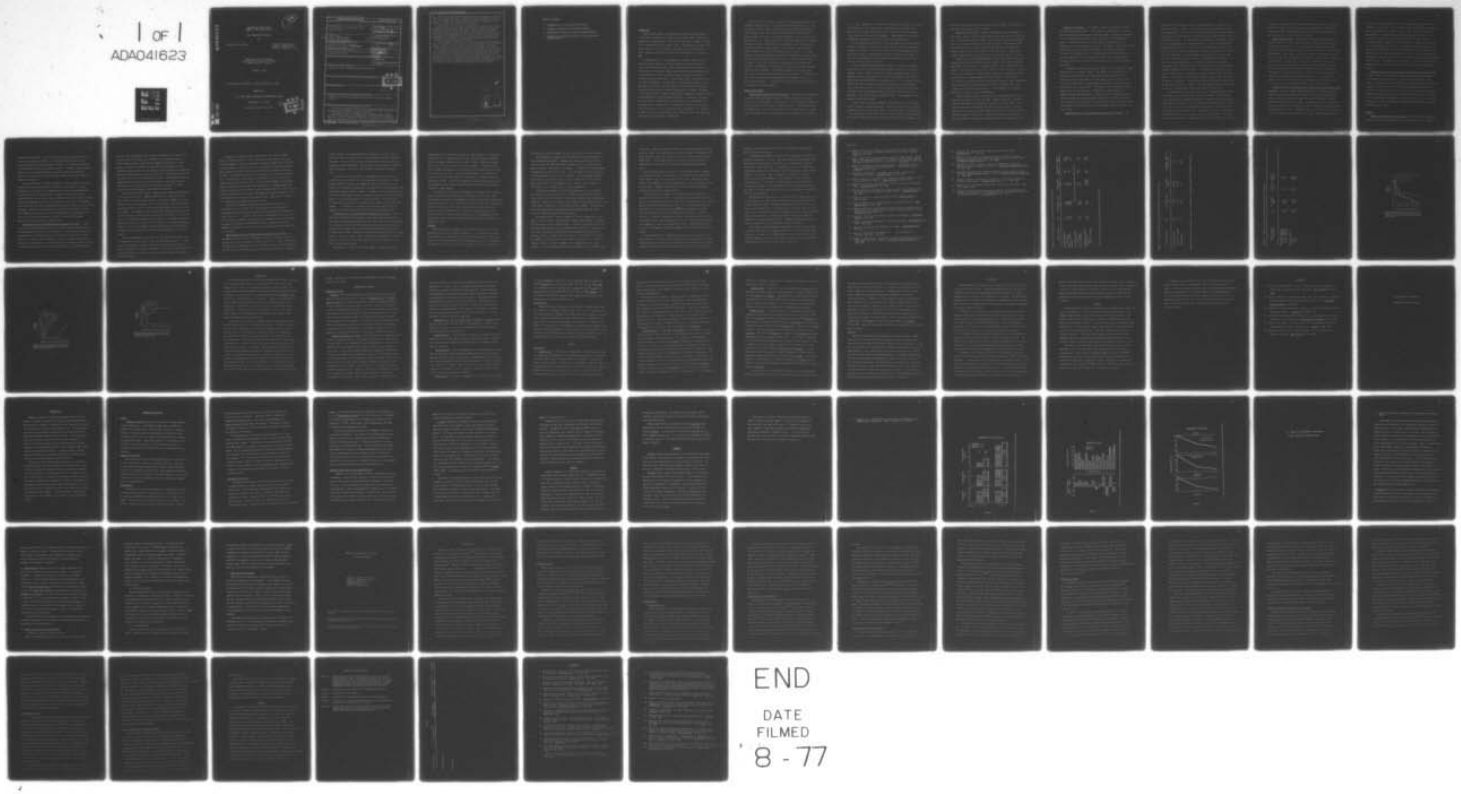

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DECONTAMINATION OF COMBAT WOUNDS IN THE INJURED SOLDIER

Final Comprehensive Report

by

Responsible Investigator: Milton T. Edgerton, M.D.
Richard F. Edlich, M.D.
George T. Rodeheaver, Ph.D.

Department of Plastic Surgery
University of Virginia Medical Center
Charlottesville, Virginia

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Management of traumatic wounds involves rapid assessment of the extent of injury and contamination. Studies in our laboratory have evaluated the interaction of antimicrobial agents and bacteria.

A fundamental tenet in the management of traumatic wounds involves successful wound cleansing. Solutions used as wound cleansers usually contain an antiseptic agent and a detergent. The majority of these detergents have been found to be toxic to tissue and impair its defenses. (con't.)

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20. Pluronic F-68 is a non-ionic detergent which was found to be non-toxic. When used to solubilize iodine, Pluronic F-68 forms a completely safe and effective iodophor. In order to formulate the most effective iodophor the parameters of pH, iodide concentration, and detergent concentration were evaluated by both "in vitro" and "in vivo" tests.

The assessment of a bacteria's susceptibility to certain antibiotics is performed with the standardized Kirby-Bauer technique. Results from the procedure are obtained 38-52 hours after a sample is taken. In critical situations results are needed much sooner. Some physicians advocate sensitivity testing directly from the clinical specimen. After evaluating the variables of inoculum size, incubation time, tissue binding and mixed organisms, it was determined that direct antibiotic sensitivity testing is a reproducible technique. In certain cases the results can be obtained within seven hours of obtaining the specimen.

Topical proteolytic enzymes have been shown to enhance the efficacy of delayed antibiotic therapy. Further studies indicate that the use of topical enzymes does not inhibit tissue defenses or wound healing. "In vitro" studies do suggest that the enzyme solutions inhibit white cell function.

The results of interaction of tissue and bacteria can be predicted on the basis of quantitative levels. Standardized and reproducible techniques have been developed for quantitating the number of aerobic organisms in fluids and tissues. These types of procedures are not available in the case of anaerobic bacteria. A procedure has been developed which employs an anaerobic homogenization chamber, pre-reduced dilution fluid, and anaerobic agar. This technique was utilized in monitoring the microbiology of burn wounds. No strict anaerobes were found after examining 105 burn wound biopsies.

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INTRODUCTION

Every traumatic wound is contaminated by bacteria and foreign debris. If the number of bacteria in a wound is large enough (10^5), infection will develop after primary closure. The type of foreign debris lodged in a wound varies considerably with the type of injury. Foreign bodies may impair the wound's ability to resist infection; the magnitude of this deleterious effect depends on the chemical and physical configuration of the foreign body.

A fundamental tenet in the management of traumatic wounds involves successful wound cleansing. A variety of agents have been employed to cleanse the contaminated wound. Some surgeons employ saline to wash the contaminants from the wound. Others employ commercially available surgical scrub solutions to cleanse the wound. These scrub solutions contain an antiseptic agent and a detergent. The antiseptic agent in the solution kills the viable contaminants. The detergents in these solutions have a distinct advantage over saline in that they reduce the surface tension between the contaminants and the wound, thereby facilitating their removal. Detergents can also minimize the frictional forces between a sponge and the wound, limiting tissue damage while ensuring wound cleansing (1). In addition, they solubilize relatively insoluble particles encouraging their dislodgement from the wound surface. Unfortunately, the detergents employed in many surgical scrub solutions are toxic to tissue and impair its defenses (2). Treatment of wounds with these detergents potentiates infection rather than protecting them against the development of suppuration.

Structure-toxicity studies performed in our laboratory have identified a nonionic detergent that is a safe and effective wound cleanser (3). This detergent is Pluronic F-68, a Pluronic polyol with a high ethylene oxide content (80 per cent) and a molecular weight of 8,350. The efficacy of Pluronic F-68 is related to its detergent properties since it has not antibacterial activity. Topical application of high concentrations of this detergent does not impair tissue defenses or wound healing (1). Scrubbing contaminated wounds with sponges soaked in this detergent ensures bacterial removal without tissue injury and protects against infection. This same detergent has been administered intravenously to human subjects without side effects, providing strong evidence for its use in man as a wound cleanser. The chemical structure of this nonionic detergent offers one additional advantage. The detergent can solubilize the relatively insoluble elemental iodine to form an iodophor. This complex solution has the antibacterial activity of iodine and the cleansing properties of Pluronic. The purpose of this study is to describe the formulation of this iodophor for use as a skin and wound cleanser. In vivo and in vitro test results that substantiate its therapeutic value are reported.

MATERIAL AND METHODS

Preparation of Iodine-Containing Solutions. One of the major impediments in the formulation of iodine solutions is the low solubility of elemental iodine in water (0.355 gm/L). Iodine solubility can be enhanced by complexing the iodine, making it more stable in aqueous solution. The most common form of complexation is the use of the iodide ion as in Lugol's solution. Iodide reacts with iodine to form a soluble complex in accordance with the equation:

$I_2 + I^- \rightleftharpoons I_3^-$. Complexation of iodine with surface active agents also increases its solubility with the iodine being incorporated within molecular aggregates of the surfactant called micelles (4). Solutions of iodine prepared in this manner are referred to as iodophors. Iodine solubility can also be enhanced by the addition of alcohol to form a tincture of iodine.

A 1 per cent aqueous solution of iodine was prepared by dissolving iodine (1.00 gm, 3.94 mM) in water containing sodium iodide (1.00 gm, 6.67 mM) and diluting it to 100 ml. A 1 per cent tincture of iodine was accomplished by dissolving 1.00 gm of iodine (3.94 mM) in 50 ml of 95 per cent ethanol. To this solution was added 1.00 gm (6.67 mM) of sodium iodide and the mixture diluted to 100 ml with distilled water.

Pluronic F-68 (BASF Wyandotte Corp, Wyandotte, Michigan) is a surface-active agent that can be used to solubilize iodine resulting in a Pluronic iodophor. Pluronic F-68 is synthesized by adding propylene oxide to the two hydroxyl groups of a propylene glycol nucleus. To the ends of this hydrophobic base are added hydrophilic ethlene oxide units. In the case of Pluronic F-68 the hydrophilic groups are controlled in length to constitute 80 per cent of the final molecule. This polar molecule orients itself with respect to iodine so that the hydrophobic portion surrounds the iodine while the remainder of the molecule forms a hydrophilic shell. This hydrophilic shell makes the iodine water soluble.

In this study two Pluronic iodophors that differed only in the concentration of sodium iodide were prepared. Initially, both solutions were made by adding 10 gm of Pluronic F-68 to 80 ml of distilled water. In one solution, 1.)) gm (3.94 mM) of iodine and 1.00 gm (6.67 mM) of sodium iodide were added. To the other, tenfold more sodium iodide (10.00 gm, 66.7 mM) was added to the

solution with a similar amount of iodine (1.00 gm, 3.94 mM). After solubilization, each solution was diluted to 100 ml.

Iodophors containing polyvinylpyrrolidone (PVP) were also prepared for this study. Iodine is rapidly solubilized by PVP without the aid of sodium iodide. The pharmacodynamics of solubilizing iodine with PVP are more complex than is the reaction between Pluronic F-68 and iodine. When iodine and PVP are mixed together, an oxidative chemical reaction occurs that generates enough iodide to ensure solubilization of the iodine (5,6). The finished product contains approximately two thirds of the original amount of iodine.

Preparation of the PVP iodophors began by dissolving 10 gm of PVP (K-30, average molecular weight 40,000; Matheson, Coleman, & Bell, East Rutherford, New Jersey) in 80 ml of water. Elemental iodine (1.00 g,. 3/94 mM) was then added, stirred overnight, and diluted to 100 ml with distilled water. Similar solutions were prepared in which different amounts of sodium iodide (1.00 gm and 10.00 gm) were added concomitantly with the iodine. Also included in this study was an undiluted solution of Betadine* antiseptic agent as obtained from the manufacturer (Purdue Frederick Co, Norwalk, Connecticut). This commercially available antiseptic agent is a PVP iodophor that is commonly employed by surgeons as a skin wound cleanser.

Previous studies indicate that the pH of an iodine solution affects its stability and antibacterial activity (7). Therefore, solutions of Pluronic F-68 and PVP iodophors were prepared as previously described except that a buffered solution was employed as the diluent instead of water. McIlvaine buffers with PH values of 2.1 and 4.0 were made using citric acid and sodium hydrogen phosphate, respectively. A buffered solution with a PH of 7.0 was also prepared using potassium dihydrogen phosphate and sodium hydroxide.

Chemistry of Iodophors. An iodophor contains a surfactant or protein that solubilizes aqueous iodine. This solubilized iodine exists in dynamic equilibrium between complexed iodine and free iodine. The majority of iodine is in the complexed form and held within the micelles of the surfactant or protein. Outside the micelle is a small amount of free iodine that is responsible for the antibacterial activity of the iodophor. This level of free iodine remains constant through contributions from the large reservoir of iodine in the complexed form. The total amount of iodine in both the free and complexed forms is termed the available iodine. The quantity of available iodine in any solution can be determined by titration with sodium thiosulfate.

All iodophor solutions contain a small amount of free iodine that is not associated with the iodophor complex. The amount of such free iodine is determined colorimetrically from the amount of iodine extracted into an immiscible organic solvent (8). To a test tube containing 1.0 ml of test solution was added 10.0 ml of spectroquality n-heptane. This solution was agitated and equilibrated at 25° C. The amount of iodine extracted into the heptane layer was determined by its absorption at 520 nanometers on a model 6A Coleman spectrophotometer. Schmidt and Winicov (8) reported that iodine absorption was linear throughout the range of 1 to 25 mg per 100 ml. The concentration of free iodine is reported in parts per million (ppm) taking into account that iodine distributes itself between heptane and water in the ratio of 30:1.

Germicidal Activity of Iodine-Containing Solutions (in vitro). The

effectiveness of an antiseptic agent is often judged by its immediate antibacterial activity. Ideally, this antibacterial activity should persist for a prolonged period. An *in vitro* test has been devised that provides a relative indication of the time interval in which an antiseptic agent exhibits effective antimicrobial action. This test measures the capacity of an antiseptic agent to kill microorganisms for an extended period of time when the contaminants are added sequentially (9). In this test, an eighteen hour nutrient broth culture was centrifuged and the bacterial sediment was washed twice in saline and then resuspended in an enriched AOAC phenol coefficient broth so that the final concentration of bacteria was 2.0×10^9 Eschericia coli/0.2ml. In the first series of experiments, the broth was prepared by boiling 5.0 gm of beef extract (DIFCO), 5.0 gm of sodium chloride, and 100 gm of peptone (DIFCO) in 900 ml of distilled water. In a second set of experiments, the broth was prepared so as to contain ten times the amount of beef extract and peptone. The protein-enriched broth was used to accelerate the inactivation of some of the iodophors. After cooling, the pH of the solution was adjusted to 6.8 and then diluted to 1,000 ml. The broth solution was then sterilized by heating it to 250° under 15 pounds of pressure for 20 minutes.

At designated time intervals, 0.2 ml of this contaminated broth solution was added to 1.0 ml of a test sample of iodine solution. At a specified time interval after each sequential addition, a sample of the test solution was removed with a bacterial culture loop (4mm in diameter) and subcultured in 1.0 ml of nutrient broth containing sodium thiosulfate (1 per cent) to neutralize any transferred free iodine. This content of sodium thiosulfate has no inhibitory effect on the growth of E coli in nutrient broth. All subcultures were incubated for three days at 37° C and then checked for turbidity.

If there was some question as to whether a solution was turbid, the nutrient broth was subcultured on nutrient agar that was subjected to an overnight incubation at 37°C. The presence of bacterial colonies on the agar plates was repeated twenty times for each test solution.

Rate of bacterial kill. The purpose of this phase of the study is to determine the rates at which iodine-containing solutions can sterilize a large inoculum of bacteria in vitro. Triplicate tubes containing either 0.2 or 0.5 ml of either aqueous iodine, tincture of iodine, or Pluronic F-68 were employed in this study. Each solution contained 1 per cent available iodine. To each tube was added 1.0 ml of phenol coefficient broth containing 6×10^{10} ℮ coli. At specified time intervals a measured aliquot of the solution was removed utilizing a calibrated loop and immediately placed in 5.0 ml of 0.9 per cent saline containing sodium thiosulfate (1 per cent). The number of viable bacteria present in each test loop was determined by standard microbiologic dilution and plating technics. This sequential monitoring of the viable counts of the treated solution provides an indication as to the rate at which the antiseptic agent kills the bacteria.

Antiseptic Activity of Iodine-Containing Solutions in Contaminated Wounds (in vivo). New Zealand albino rabbits weighing 2.4 to 3.0 kg were anesthetized with pentobarbital sodium (33 mg/kg). Their backs were clipped with electric shears, depilated with Surgex, and washed thoroughly with water followed by 70 per cent ethyl alcohol. Using aseptic technic, standardized surgical incisions (2 cm in length) were made through paravertebral skin down to the panniculus carnosus. Randomized wounds on each of the rabbits received 0.1 ml of designated iodine-containing solutions. Solutions evaluated were

tincture of iodine, Betadine antiseptic solution, and two Pluronic F-68 iodophors; all contained 1 per cent sodium iodide, and the Pluronic iodophors were buffered separately to pH 2.1 and 4.0. After the iodine solutions had remained in the wounds of designated rabbits for either 0.5, 1, 2, or 3 hours, the wounds were contaminated with 2.8×10^5 E coli/0.02 ml. In a separate group of rabbits, 0.1 ml of the Pluronic iodophor buffered to pH 2.1 was introduced into the wound. At intervals of 2,3,4, and 5 hours, six wounds were inoculated with 2.8×10^5 E coli/0.02 ml. Thirty minutes after contamination, each wound was swabbed with a sterile cotton-tipped applicator. The applicator was then immersed in 5 ml of saline containing sodium thiosulfate (1 per cen w/v). The applicator was vortexed for 1 minute and the number of organisms present in the solution was determined by standard microbiologic technics.

Antiseptic Activity of Iodine-Containing Solutions on Contaminated Skin. The paravertebral skin of anesthetized rabbits was depilated and prepared with iodine-containing solutions. A measured volume (0.1 ml) of either tincture of iodine or Pluronic F-68 (1 per cent of sodium iodide, buffered to a pH of 2.1) was placed on the rabbit's back and spread uniformly over a 3 cm^2 area of skin with a glass rod. At different time intervals, selected areas were contaminated with 0.02 ml of saline containing 1.4×10^7 E coli. Thirty minutes after contamination, the test sites were excised. The number of bacteria in each skin sample was determined by quantitative tissue microbiologic technics (10).

RESULTS

Chemistry of Iodine-Containing Solutions. PVP was used to solubilize iodine in aqueous solution without the addition of sodium iodide. (Table 1).

However, only about 60 per cent of the original iodine was accounted for by titration with sodium thiosulfate. Initially PVP reacted chemically with iodine to generate carbon-iodine bonds and iodide. This iodide then assisted in normal solubilization of the remaining iodine. In contrast, Pluronic polyols did not react in an oxidative manner with iodine to generate iodide and, therefore, did not readily solubilize aqueous iodine unless sodium iodide was added to the solution.

Use of sodium iodide in preparing iodophors not only enhanced the solubilization of iodine but also affected the amount of free iodine in solution. The amount of free iodine in the iodophor solution was inversely proportional to the concentration of sodium iodide. Iodophors of PVP without sodium iodide contained more than three times the concentration of free iodine (1.8 ppm) than the same iodophor with 1 per cent sodium iodide (0.5 ppm). PVP iodophors containing 10 per cent sodium iodide had extremely small quantities of free iodine (0.2 ppm). Similarly, the concentration of free iodine (2.5 ppm) in Pluronic F-68 iodophors containing only 1 per cent sodium iodide was considerably more than the free iodine concentration (0.3 ppm) in Pluronic F-68 iodophor solutions containing 10 per cent sodium iodide.

Germicidal Activity of Iodine-Containing Solutions (in vitro). In this test, heavily contaminated phenol coefficient broth was added sequentially to an iodine-containing solution every 60 seconds. Fifteen and 30 seconds after contamination, the antiseptic agent was cultured for the presence of viable bacteria. If bacteria were not detected in the iodine-containing solution 15 seconds after contamination, this was evidence for an immediate concentration of free iodine that was sufficient to destroy the bacteria. When bacteria were

cultured from the antiseptic agent solution 15 seconds after inoculation but were not present 30 seconds after inoculation, this suggested that the initial concentration of iodine was not adequate to eliminate all viable bacteria. However, the absence of viable bacteria 30 seconds after contamination suggested that sufficient amounts of iodine were being released over an extended time period to sterilize the bacteria. The germicidal activity of the iodine solutions was also judged by the number of sequential additions of contaminated broth solutions that were necessary to inactivate the iodine. Inactivation of iodine solution was considered to be present if viable bacteria were cultured from the iodine-containing solution.

The free iodine concentration of iodophors was an important determinant of its antibacterial activity. Iodophor solutions with a high level of free iodine rapidly eliminated viable bacteria. In contrast, iodophors with low levels of free iodine exhibited a slower rate of bacterial kill but displayed prolonged antibacterial activity due to the slow release of free iodine from the iodophor. PVP iodophor, without sodium iodide, displaying high levels of free iodine rapidly eliminated bacteria even after the addition of twenty aliquots of the contaminated peptone broth. (Table I). PVP iodophors containing sodium iodide failed to sterilize the first addition of contaminated peptone broth at either 15 or 30 seconds. Betadine effectively sterilized bacteria 30 seconds after inoculation but failed to achieve a complete kill within 15 seconds.

Pluronic F-68 iodophor containing 1 per cent sodium iodide rapidly sterilized each of more than twenty aliquots of broth added sequentially. Addition of larger amounts of sodium iodide (10 per cent) severely limited its germicidal capacity. Pluronic F-68 iodophors containing high concentrations of sodium iodide did not sterilize any of the aliquots of sequentially added contaminated broth.

As expected, the aqueous iodine solutions and the tincture of iodine rapidly elimminated bacteria. It was interesting to note, however, that they like the iodophors, resisted inactivation of sequential addition of aliquots of contaminated broth. Identification of the iodine-containing solution with the most pronounced antiseptic activity was facilitated by increasing the content of protein in the phenol coefficient broth employed in the germicidal capacity test. Utilizing this enriched medium the antiseptic activity of solution of aqueous iodine and tincture of iodine, a Pluronic F-68 iodophor, was measured. When challenged with the enriched broth and bacteria, the noncomplexed iodine solutions lost their activity after thirteen sequential additions (Table II). The germicidal capacity of the Pluronic F-68 iodophor was apparent beyond the twentieth addition of an aliquot of contaminated broth. These results indicated that iodophors have prolonged germicidal activity.

The pH of the iodophor solution was an important determinant of its antibacterial activity. (Table III). When the pH of the solution was 2.1, the iodophor exhibited a greater degree of activity than did the iodophors with a pH of 4.0 or 7.0. The ability of Pluronic F-68 iodophors with a low level of free iodine (0.3 ppm) to sterilize bacterial contamination within 30 seconds was apparent only when the solution had a low pH. Likewise, only the PVP iodophor with a pH of 2.1 was able to sterilize the additions of bacteria within 15 seconds.

Rate of Bacterial Kill of Iodine-Containing Solutions (in vitro). In noncomplexed iodine solutions, like aqueous iodine and tincture of iodine, all the iodine was free with immediate antibacterial activity. When a large volume (0.5 ml) of either aqueous or tincture of iodine was added to broth containing 6×10^{10} E coli, all the bacteria were killed within 20 seconds. If a smaller

volume (0.2ml) of the noncomplexed iodine was added to a similar level of inoculum in broth, the bacterial count was immediately reduced to 10^4 within 30 seconds. After this time interval, the bacterial count of the solution remained essentially unchanged. This persistent level of bacteria indicated that the antiseptic activity of these solutions lasted short periods of time.

The speed with which the Pluronic F-68 iodophor killed bacteria was significantly less than that for the noncomplexed iodine solutions. Larger volumes (0.5ml) of Pluronic F-68 iodophor killed all the bacteria in the broth within 90 seconds as compared with 15 seconds for a similar volume of the noncomplexed iodine solutions. However, the capacity of small volumes (0.2 ml) of Pluronic F-68 iodophor to sterilize bacteria was superior to that of similar amounts of either aqueous iodine or tincture of iodine. Within 2 minutes, all bacteria were killed after treatment with this quantity of Pluronic F-68 iodophor. It is important to reiterate that a similar volume of the non-complexed iodine solutions failed to sterilize the contaminated broth.

Antiseptic Activity of Iodine-Containing Solutions (In vivo). The non-complexed iodine-containing solutions provided some degree of protection of the wounds from subsequent contamination for a short time interval (30 minutes). It is important to note that these solutions never completely eradicated the inoculum that was added to the standardized experimental wound. Low levels of viable bacteria were recovered from the wounds pretreated with either aqueous iodine or tincture of iodine. When the wound was contaminated 60 to 120 minutes after treatment with these antiseptic solutions, no reduction in the level of bacteria was apparent.

The superiority of the Pluronic F-68 iodophor as compared with these

noncomplexed iodine-containing solutions was readily apparent. Treatment of the wound with this iodophor buffered to pH 2 provided complete protection against subsequent contamination for 3 hours. Bacteria added to the wound during this time interval were killed by the iodophor. Germicidal activity of the iodophor was apparent for at least another 60 minutes after this time, but it was not sufficient to kill all the contaminants. As the pH of the solution of Pluronic F-68 iodophor was increased, its therapeutic effectiveness was diminished. The Betadine iodophor provided more protection than did the noncomplexed iodine-containing solution but significantly less than did the Pluronic F-68 iodophors.

The success of the Pluronic F-68 iodophor was also apparent on skin subjected to subsequent contamination with high doses of E coli (1.4×10^7). Pretreatment with Pluronic F-68 iodophor protected the skin from a challenge of surface contamination for at least 5 hours. Thirty minutes after contamination with E coli during this time interval, quantitative cultures of the skin failed to identify viable bacteria. Tincture of iodine provided minimal protection against subsequent skin contamination. This noncomplexed iodine solution was rapidly inactivated by skin and did not exhibit prolonged antibacterial activity.

COMMENTS

Iodine has long been regarded as an excellent germicide, buts its use has been restricted by its physical properties: its low solubility in water (0.355 gm/L) and its high volatility. The vapors generated from iodine solutions are irritating and sensitizing to eyes, mucosa, and skin. Increasing the solubility of iodine with the use of alcoholic solvents or the addition

of sodium iodine does not reduce the toxic side effects of volatile iodine.

The development of iodophors has eliminated the problems encountered in using noncomplexed solutions. Iodophors are aqueous solutions of iodine in which the iodine has been solubilized and stabilized with a surface-active agent or protein. The iodine in an iodophor is in dynamic equilibrium between a "complexed" form and a "free' form. It is the amount of iodine in the free form that governs the activity o the iodophor. Highly complexed iodophors, which have very little free iodine, are very stable, do not stain, have no odor, and are nonirritating to human tissue. However, this low level of free iodine is still highly effective in killing bacteria.

The ability of iodophors to maintain prolonged activity is a result of slow release of iodine from a carrier. Iodine release occurs only when the steady state level of free iodine is depleted by reaction with a contaminating substance. Therefore, the parameters that regulate the equilibrium value of free iodine should be paramount in the formulation of an iodophor. The parameters of greatest importance are concentration of surfactant, amount of iodine, content of iodide, and pH of the final solution.

The concentration of iodine in solution is directly proportional to the amount of the surfactant. As surfactant is added to the solution, it combines with the iodine to form a soluble complex. The pH of the solution affect both the bactericidal properties of the iodophor and its stability. Iodine reacts with water to produce various hydrated species depending on the pH of the solution (11,12). In highly acidic solutions all of the iodine is titratable as elemental iodine (I_2) which has the highest biocidal activity. The high content of elemental iodine in iodophor with a low pH accounts for their success in sterilizing contaminated tissue as compared with the iodophors with

a higher pH. Lowering the pH also stabilizes the system and reduces the loss of active iodine. As the pH increases the iodine changes into hypoiodous acid (HIO), hypoiodate (IO-), and finally to iodate ion (IO_3-) when the solution becomes basic. The antiseptic activity of the basic iodine solutions is considerably less than that of the acidic solutions containing elemental iodine.

One of the most important parameters in the formulation of an iodophor is the content of iodide in the solution. Once iodine has been solubilized, iodide regulates the equilibrium value of free iodine. Small additions of iodide to the iodophor increase the complexation of iodine, reducing the level of free iodine. Iodophors with low levels of free iodine have prolonged germicidal activity and increased stability.

The speed with which iodophor and noncomplexed iodine solutions kill bacteria is considerably different. In an aqueous iodine solution all of the iodine is in the free form and is available for instantaneous reaction with both bacteria and other proteinaceous material. If the iodine solution does not eliminate all of the bacteria within these first few seconds, no further significant bacterial kill will be observed since there is no residual activity. In contrast, iodophors have a slower but sustained rate of action that allows for more efficient germicidal activity. The level of free iodine in an iodophor can easily be increased by dilution. The addition of saline or water to an iodophor results in an iodophor solution that can kill bacteria rapdily.

The pharmacodynamics of iodophors make them especially valuable as skin wound cleansers in surgery. They afford immediate sterilization of the tissue and prolonged protection against subsequent bacterial contamination. The non-complexed iodine solutions are inactivated rapidly during their application.

Although they afford rapid bacterial kill, they do not protect the wound against further contamination.

In developing an iodophor, the selection of the protein or surfactant to solubilize the iodine is critical. For use in human subjects, Pluronic F-68 has many distinct advantages. Long-term toxicity studies have indicated that Pluronic F-68 is safe (13). The successful use of this polyol as an intravenously administered emulsifying agent in human subjects lends further support to its use (14). When it complexes with iodine, the chemical structure of Pluronic F-68 appears to be unchanged since the reaction is easily reversible. In contrast, a significant portion of the PVP binds irreversibly to iodine. PVP reacts with iodine to form carbon-iodine bonds and iodide. The exact nature and toxicity of this new polymeric compound(s) have not been determined.

PVP has had other applications in clinical medicine. German experience at the Russian front in World War II established the effectiveness of PVP in the treatment of shock. The demonstration that 35 to 49 per cent of the PVP was retained indefinitely in the body after intravenous injection stimulated study of the deleterious effects associated with this storage phenomenon. Numerous clinical reports (15-22) have noted the formation of cutaneous lesions after the administration of PVP. Although the lesions are not precancerous, they do result in considerable cosmetic deformity.

The cutaneous storage phenomenon encountered after the systemic administration of PVP reflects the kidneys' inability to excrete molecular weight fractions of PVP greater than 40,000 daltons. Since the commercially available PVP iodophors contain molecular weight fractions greater than 40,000, it would seem prudent to restrict their use to intact skin.

References

1. Rodeheaver GT, Smith SL, Thacker JG, Edgerton MT, Edlich RF: Mechanical Mechanical cleansing of contaminated wounds with a surfactant, Am J Surg 129: 241, 1975.

2. Custer J, Edlich RF, Prusak M, Madden J, Panek P, Wangensteen OH: Management of the contaminated wound V. An assessment of the effectiveness of pHisoHex and Betadine surgical scrub solutions. Am J Surg 121: 572, 1971.

3. Edlich RF, Schmolka IR, Prusak MP, Edgerton MT: The molecular basis for toxicity of surfactants in surgical wounds. I. EO-PO block polymers. J Surg Res 14: 277, 1973.

4. Allawale NA, Riegelman S: The properties of iodine in solutions of surface-active agents. J Am Pharm Assoc 42: 396, 1953.

5. Hugo WB, Newton JM: The solubility of iodine in aqueous solutions of non-ionic surface-active agents. J Pharm Pharmacol 15: 731, 1963.

6. Shelanski HA, Shelanski MV: PVP-iodine: history, tocicity, and therapeutic uses. J Int Coll Surg 25: 727, 1956.

7. Hugo WB, Newton JM: The stability, staining and corrosive properties of an iodine non-ionic surface-active agent complex. J Pharm Pharmacol 16: 173, 1964.

8. Schmidt W, Winicov M: Detergent/iodine systems. Soap Chem Spec 43: (8), 61, 1967.

9. Cantor A, Shelanski HA: A "capacity" test for germicidal action. Soap Sanitary Chem 27: 133, 1951.

10. Madden JE, Edlich RF, Schauerhamer R, Prusak M, Borner J, Wangensteen OH: Application of principles of fluid dynamics to surgical wound irrigation. Curr Top Surg Res 3: 85, 1971.

11. Chang SL: The use of active iodine as a water disinfectant. J Am Pharm Assoc 47: 417, 1958.

12. Wilson JL, Mizuno WG, Bloomberg CS: Iodine disinfectants. Soap Chem Spec 36 (12): 100, 1960.

13. Leaf CW: Toxicology of some non-ionic surfactants. Soap Chem Spec 43: 48, 1967.

14. Cohn I Jr, Singleton S, Hartwig QL, Atik M: New intravenous fat emulsion, JAMA 183: 755, 1963.

15. Dupont A, Lachapelle JM: Dermite due a un depot medicamenteux au cours du traitement d'un diabete insipide. Bull Soc Fr Dermatol Syphiligr 71: 508, 1964.

16. Lachapelle JM: Thesaurismose cutanee par polyvinylpyrrolidone. Dermatologica 132: 476, 1966.

17. Bazek A, Geraud J, Guilhem A, Dupre A, Rascol A, Cantala P: Maladie de Dupont et Lachapelle (Thesaurismose cutanee par polyvinylpyrrolidone). Arch Belges Dermatol Syphilgr 22: 227, 1966.

18. LeCoulant P, Texier L, Leuret J, Geniaux: Iconographie d'un cas de thesaurismose par posthypophyse polyvidone. Bull Soc Fr Dermatol Syphiligr 74: 675, 1967.

19. Lachapelle JM, Bourlond A: Etude en microscopie electronique de la thesaurismose cutanee par polyvinylpyrrolidone. Bull Soc Fr Dermatol Syphiligr 74: 538, 1967.

20. Cabanne F, Chapuis JL, Duperrat B, Putelat R: L'infiltration cutanee par la polyvinylpyrrolidone. Ann Anat Pathol (Paris) 11: 385, 1968.

21. Bastien HG: L'infiltration tissulaire par la polyvinylpyrrolidone. These n° 9 (Dijon), 1969.

22. Thivolet J, Leung TK, Duverne J, Leung J, Volle H: Ultrastructural morphology and histochemistry (acid phosphatase) of the cutaneous infiltration by polyvinylpyrrolidone. Br J Dermatol 83: 661, 1970.

TABLE 1 Germicidal Capacity Test with Phenol Coefficient Broth

Iodine Solution	pH	Available Iodine (%)	Free Iodine (ppm)	Germicidal Capacity 15 sec	Germicidal Capacity 30 sec
10% PVP Iodophors					
1% I_2	2.3	0.62	1.8	>20	>20
1% I_2 + 1% NaI	3.1	0.93	0.5	0	ICR*
1% I_2 + 10% NaI	2.9	0.89	0.2	0	0
Betadine	4.0	0.88	0.8	0	>20
10% F-68 Iodophors		(Part of 1 gm of I_2 was not solubilized)			
1% I_2					
1% I_2 + 1% NaI	4.4	0.94	2.5	>20	>20
1% I_2 + 10% NaI	4.4	0.89	0.3	0	0
Noncomplexed Iodine					
1% I_2 + 1% NaI	6.0	1.00	10,000	>20	>20
Aqueous	5.8	1.00	10,000	>20	>20
50% ethyl alcohol					

*ICR= Inconsistent results due to borderline free iodine level.

TABLE II Germicidal Capacity Test with Enriched Phenol Coefficient Broth

Iodine Solution	pH	Available Iodine (%)	Free Iodine (ppm)	Germicidal Capacity* 2 minute test
Aqueous Iodine	6.0	1.00	10,000	13
Tincture of Iodine	5.8	1.00	10,000	13
F-68 Iodophor	2.1	0.99	2.7	>20

*Reported as last sterile tube in a series of twenty tubes, indicating limits of antibacterial activity.

TABLE III Influence of pH on the Germicidal Capacity of Iodophors

Iodine Solution (buffered)	pH	Free Iodine (ppm)	Germicidal Capacity 15 Sec	30 Sec
10% F-68 Iodophors				
1% I_2 + 10% NaI	2.1	0.3	0	>20
1% I_2 + 10% NaI	4.0	0.3	0	0
1% I_2 + 10% NaI	7.0	0.3	0	0
10% PVP Iodophors				
1% I_2	2.1	1.8	>20	>20
1% I_2	4.0	1.8	0	>20
1% I_2	7.0	1.8	0	>20

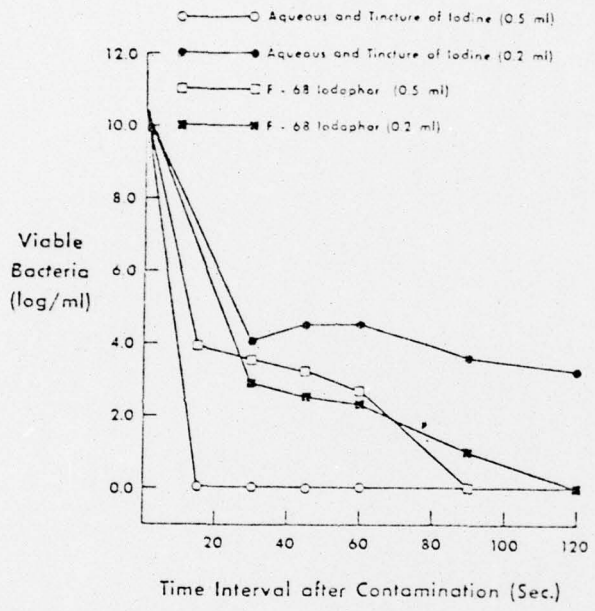

Figure 1. Rate of bacterial kill of iodine-containing solutions (in vitro). Pluronic F-68 iodophors exhibited more prolonged antibacterial activity (in vitro) than did the noncomplexed iodine solutions.

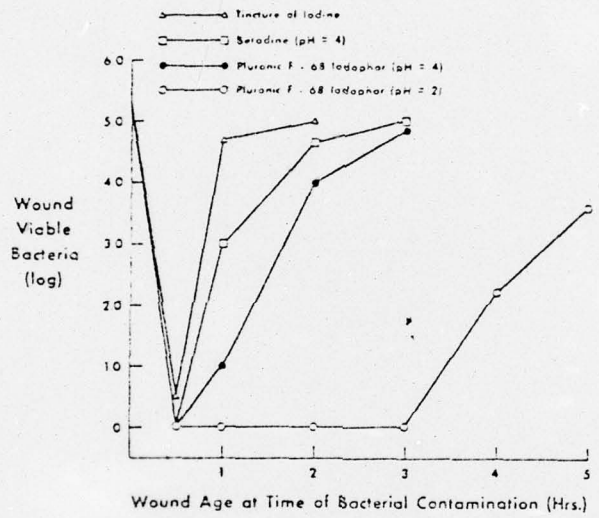

Figure 2. Antiseptic activity of iodine solutions in wounds. Pluronic F-68 iodophors protected wounds against subsequent contamination for 3 hours.

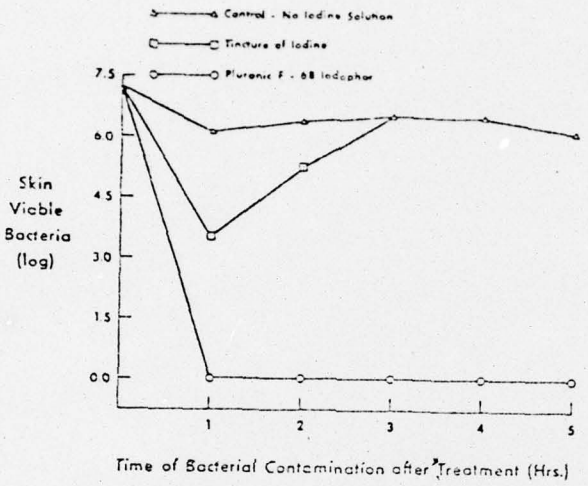

Figure 3. Antiseptic activity of iodine solutions on skin. Pluronic F-68 iodophors protected skin against subsequent contamination for at least 5 hours.

INTRODUCTION

The Food and Drug Administration has recommended the Kirby-Bauer technique as the standardized procedure for the determination of antimicrobial disc susceptibility.[3] This is a standardized technique in which variables like culture medium, inoculum type and size, and concentration and stability of the antibiotic are well controlled.[1] General acceptance of the *in vitro* susceptibility test has been aided by its simplicity. However, the prolonged time interval (38 to 52 hours) between receiving the specimen for culture and completion of the antibiotic disc sensitivity test has remained a distinct disadvantage. Physicians are often frustrated by the delay since it postpones treatment with the appropriate antimicrobial drug, leading in some cases to increased mortality and morbidity.

In these clinical situations, reliable results are needed more quickly than can be obtained with this conventional technique. In order to circumvent this delay, physicians have advocated antibiotic sensitivity testing directly on the clinical specimen. When such a rapid test is employed, many of those variables are no longer controlled. The inoculum size may vary considerably. The presence of mixed cultures may be a limiting factor. The binding of the antibiotic to the tissue may lead to an erroneous interpretation of the results. A reduction in the incubation time requirements in the standard Kirby-Bauer test may also influence the reliability of the results. The purpose of this study was to analyze critically the influence of each of these determinants on the reliability of antibiotic sensitivity tests performed directly on clinical specimens. This study demonstrates that the bacterial susceptibilities to antibiotics as determined by the modified test correlated extremely well with those obtained by the standard Kirby-Bauer

method. The potential of this modified rapid method as a guide to *in vivo* therapy is discussed.

MATERIALS AND METHODS

Experimental Study

Bacteria--The organisms employed in this study were obtained from the American Type Culture Collection. Strains of *Escherichia coli* and *Staphylococcus aureus* were selected because they are common pathogens accounting for burn wound sepsis and soft tissue infections. The bacteria were maintained on tryptose blood agar plates. Prior to each experiment the bacteria were transferred by sterile loop into 25 ml of trypticase soy broth. The broth culture was agitated at 37° C for eighteen hours before the bacteria were sedimented by centrifugation. After decanting the broth supernate, the bacterial sediment was washed twice in 0.9% saline and the final sediment resuspended in 2.0 ml of 0.9% saline. The number of organisms in the suspension was quantitated by routine serial dilution and plating techniques.

Antibiotic Susceptibility Test-- The test procedure was essentially that of the Kirby-Bauer technique. Test plates were prepared by filling sterile disposable petri dishes (150 mm) with Mueller-Hinton agar to a depth of 5 mm. These plates were enclosed in plastic bags and stored in the refrigerator until used. At the time of testing, a bacterial suspension of known concentration was streaked onto the surface of the agar in three directions using sterile cotton swabs. After a three minute delay, antibiotic discs were applied to the agar surface with an automatic dispenser and pressed to the surface with sterile forceps. During each test, the following eleven antimicrobial agents were used: ampicillin, bacitracin, chloramphenicol, gentamycin, cephalothin, neomycin, penicillin G, polymyxin B, tetracycline, streptomycin and colymycin. After incubation at 37° C for eighteen hours,

the zone of inhibition around each disc was measured with a ruler on the undersurface of the petri dish. The endpoint was taken as the complete inhibition of growth as determined by the naked eye. The zone diameters as recommended by the Food and Drug Administration were used to interpret the susceptibility of the bacteria to antibiotics. The susceptibility of the organism was considered to be either sensitive, intermediate or resistant according to the size of the zone of inhibition. Six plates were prepared for each measurement. The influence of the following variables on the interpretation of the sensitivity of bacteria to antibiotics was determined in the next series of experiments.

Inoculum Size-- Bacterial suspensions of E. coli or S. aureus were prepared to contain 10^5, 10^6, 10^7, 10^8, or 10^9 organisms/ml. Mueller-Hinton plates were streaked with the various concentrations of organisms and a routine susceptibility test performed.

Incubation Time-- Plates from the test above were removed from the incubator after sever hours and the zones of inhibition recorded. The plates were returned to the incubator and reexamined after the standard eighteen hours of incubation.

Tissue Binding-- Bacterial suspensions were prepared as before, except that the suspensions contained 10% (w/v) homogenized tissue. The tissues employed were skin, muscle, or burn eschar. Suspensions of 10% tissue were very viscous and probably would be the maximum amount of tissue ever exposed to an antibiotic test plate. The antibiotic sensitivity of these bacterial suspensions was compared to that of bacterial suspensions without tissues after a seven and eighteen hour incubation.

Mixed Cultures-- The effect of E. coli on the interpretation of sensi-

tivity of S. aureus to antibiotics was evaluated by preparing suspensions of 10^8 S. aureus which also contained 10^4, 10^5, 10^6, 10^7, or 10^8 E. coli. This experiment was then repeated except suspensions of 10^8 E. coli were prepared which also contained 10^4, 10^5, 10^6, 10^7, or 10^8 S. aureus. The susceptibility of each of these bacterial suspensions to antibiotics was recorded by the standard Kirby-Bauer test.

Clinical Study

Homogenized clinical specimens of burn eschar or infected wound tissue were subjected to immediate antibiotic sensitivity testing. In addition to the eleven antibiotics employed in the experimental study, another seven clinically important antibiotics were used: carbenicillin, erythromycin, kanamycin, nafcillin, oxacillin, tobramycin, and vancomycin. In addition, the number of viable bacteria in the homogenate was quantitated by standard serial dilution and plating techniques. Bacteria isolated from the quantitative measurements were identified and the individual antibiotic susceptibility of each isolated organisms determined by the standard Kirby-Bauer procedure.

RESULTS

Experimental

Inoculum Size-- A heavy growth of organisms on the agar plate is essential to permit interpretation of the antibiotic susceptibility. A bacterial inoculum of 10^5 or less organisms resulted in non-confluent growth on the plate in which the zones of inhibition are not readable. A non-confluent but readable growth pattern was encountered with 10^6 and 10^7 organisms. Determination of antibiotic sensitivities at this level is fortuitous, since 10^6 or more bacteria result in clinically significant wound infection and burn

wound sepsis while smaller doses of bacteria are clinically insignificant. When the inoculum delivered to the agar plate was 10^8 or greater, a confluent lawn of bacteria was readily apparent in which antibiotic sensitivity testing was easily accomplished.

When the zones were readable, inoculum size had an insignificant effect on the interpretation of antibiotic susceptibility. After an eighteen hour incubation, only small differences in zones of inhibition were observed when the inoculum was varied from 10^6 to 10^9 organisms/ml. The zone of inhibition was frequently inversely proportional to the inoculum size. Increasing the inoculum size usually resulted in a slight decrease in the diameter of the zone of inhibition. The resultant reduction in zone size did not affect the interpretation of antibiotic susceptibility in any of the 264 tests performed with E. coli and in only one of 264 tests conducted with S. aureus.

Incubation Time-- With E. coli and S. aureus, small differences in zone size were observed when the results were recorded after seven hours as compared to those measured following an eighteen hour incubation. These changes in zone size on agar plates containing E. coli influenced the interpretation of results in 14 of 264 tests. An initial reading of intermediate at seven hours was changed to resistant after an additional eleven hours of incubation. These changes were restricted to the chloramphenicol discs when the inoculum was 10^6 and 10^7 and involved only a 2 mm alteration in the diameter of inhibition. An initial reading of sensitive at seven hours which was subsequently read as intermediate accounted for three of the changes in the tests involving S. aureus. Five other changes occurred with S. aureus which fell into the intermediate range at seven hours, changing to resistant at eighteen hours. It is important

to note that readings of sensitive never changed to resistant as a result of altering the length of incubation.

Tissue Binding-- The presence of tissue (10%) in the homogenate fluid did not significantly change the interpretation of antibiotic susceptibility of either E. coli or S. aureus. Interpretation of the antibiotic sensitivity test was altered in only four of 464 tests as a result of the presence of tissue in the bacterial suspension. In these exceptions, the reading was changed from sensitive to intermediate in specimens containing eschar.

Mixed Cultures-- In mixed cultures, the interpretation of antibiotic susceptibility was related to the number of bacteria in the culture. When 10^6 or less E. coli were added to a suspension containing 10^8 S. aureus, the determination of antibiotic sensitivities of the S. aureus was easily mad and unchanged from that encountered with S. aureus alone. These levels of E. coli resulted in non-confluent growth that did not mask the inhibition zones in S. aureus. When 10^7 or 10^8 E. coli were present in the S. aureus suspension, both organisms grew to produce confluent lawns. In this case, the diameter of the inhibition zone was determined by the least sensitive organism. This is exemplified by the antibiotic susceptibility to tetracycline of mixed cultures of large numbers (10^8) of both E. coli and S. aureus. In these mixed cultures, the clear zone of inhibition around the tetracycline disc measured 6 mm comparable to that in a culture of only E. coli. This size of the zone of inhibition due to tetracycline on plates with E. coli was considerably smaller than that (28 mm) encountered on plates subjected to large numbers of S. aureus.

In these mixed culture experiments, we observed an interesting growth pattern that provided insight into the antibiotic sensitivity of the mixture

of organisms growing on agar plates. This growth pattern occurred when there were dramatic differences in the sizes of the zones of inhibition of the mixture of organisms as in the above case. A complete zone of inhibition was readily apparent, indicating the antibiotic susceptibility of the most resistant organism. In addition, a larger incomplete zone of inhibition was noted around the clear zone, denoting the antibiotic susceptibility of the more sensitive organism. The size of this incomplete zone of inhibition was comparable to that of the clear zone noted in the tests performed on the more sensitive organism. The organism isolated from the incomplete zone of inhibition was the most resistant organism. Similar results were obtained when a suspension of 10^8 E. coli was mixed with varying numbers of S. aureus (10^4 to 10^8). Again, the clear zone of inhibition was determined by the least sensitive organism.

Clinical

During a seven month period we conducted a preliminary study to compare the results of the direct antibiotic susceptibility test of clinical specimens with that obtained from individual isolates of the same specimen. During this interval, 80 clinical specimens were obtained which contained at least 10^6 organisms/gm of tissue. 865 antibiotic tests were performed on the isolated colonies using the standard Kirby-Bauer procedure. An agreement between these two tests was encountered in 699 (81%) of the cases. In 19% of the tests in which there was disagreement, the combination of one organism with a zone size interpreted as sensitive with another organism whose zone size was considered resistant resulted in a zone size interpreted as resistant. In no cases did we find a mixture of organisms yielding a resistant interpretation when the same individual organisms gave a zone which was judged to be sensitive.

DISCUSSION

This preliminary study demonstrated the feasibility of direct antibiotic testing of clinical specimens. The major advantage gained by this modified technique was the ability to determine antibiotic susceptibilities of the pathogen within seven hours after submission of the sample. The modification employed did not alter most of the standards recommended by the Food and Drug Administration, since there was no inclusive change in the medium, agar depth or antibiotic sensitivity disc.

The changes in the Kirby-Bauer test necessitated by using the clinical specimen rather than the isolated bacteria did not significantly alter the interpretation of the antibiotic susceptibility. When a clinically significant number of bacteria (10^6-10^9) were present in the clinical specimen, variations in the inoculum size did not appreciably change the results of the antibiotic susceptibility tests. Reducing the incubation requirement for the standard Kirby-Bauer antibiotic susceptibility test from eighteen to seven hours also did not significantly limit the accuracy of the test. The feasibility of recording the results of antibiotic susceptibility tests after this short time interval has also been demonstrated in separate studies by Liberman and Robertson[4] and Boyle et al[2] using isolated bacterial cultures.

As expected, the variables most difficult to standardize in the direct test was the heterogenous inoculum containing large numbers (10^7) of different organisms. In these cases, a zone of inhibition interpreted as sensitive with one organism was masked by the presence of the confluent growth of an organism whose zone of inhibition was considered resistant. We did not encounter the circumstance in which a number of sensitive species gave reactions interpreted as resistant when tested in combination.[6] However, even in this case it is

possible that mixed culture sensitivities may provide the most valid information in mixed infections since they most closely simulate the real clinical situation.[5] The merit of direct antibiotic sensitivity testing of clinical specimens must awair further experimental and clinical studies in which the results of the proposed sensitivity are shown to have some correspondence with the clinical situation.

SUMMARY

The purpose of this study was to develop a more rapid antibiotic sensitivity test of the pathogens in soft tissue infections and burn wound eschar. The proposed rapid antibiotic sensitivity was performed directly on the clinical specimen rather than on single strains of bacteria isolated from the tissue. Performing the antibiotic sensitivity tests directly on the tissue sample allows the physician to receive the test results seven hours after receiving the specimen rather than 38-52 hours later, a time delay encountered with the conventional technique. This modification employed did not alter most of the standards recommended by the Food and Drug Administration since there was no inclusive change in the medium, agar depth or the antibiotic sensitivity disc.

The changes in the Kirby-Bauer test necessitated by using the clinical specimen did not alter significantly the interpretation of the antibiotic susceptibility. When clinically significant numbers of bacteria (10^6-10^9) were present, variations in the inoculum size did not appreciably change the results of the antibiotic susceptibility tests. Reducing the incubation requirement for the standard Kirby-Bauer antibiotic susceptibility test to seven hours also did not significantly limit the accuracy of the test.

As expected, the variable most difficult to standardize was the heterogenous inocula containing large numbers (10^7) of different organisms. A zone of inhibition interpreted as sensitive with one organism was occasionally masked by the presence of the confluent growth of an organism whose zone of inhibition was considered resistant. However, even in this case, it is possible that mixed culture sensitivities may provide the most valid information in mixed infections, since they more closely simulate the real clinical situation.

REFERENCES

1. Bauer AW, Kirby WMM, Sherris JC, and Turck M: Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Path 45: 439, 1966.

2. Boyle JV, Fancher ME, and Ross RW: Rapid, mofified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial discs. Antimicrob Agents Chemother 3: 418, 1973.

3. Department of Health, Education, and Welfare. Food and Drug Administration: Proposed rule making. Fed Regist 36: 6899, 1970.

4. Liberman DF, and Robertson RG: Evaluation of a rapid Bauer-Kirby antibiotic susceptibility determination. Antimicrob Agents Chemother 7: 250, 1975.

5. Linn BS, and Szabo S: The varying sensitivity to antibacterial agents of micro-organisms in pure vs. mixed cultures. Surgery 77: 780, 1975.

6. Shahidi A, and Ellner PD: Effect of mixed cultures on antibiotic susceptibility testing. Appl Microb 18: 766, 1969.

III Side Effects of Topical

Proteolytic Enzyme Treatment

INTRODUCTION

Immediate treatment of heavily contaminated wounds with either topical or systemic antibiotics can reduce the wound infection rate. When antibiotic treatment is delayed for three or more hours, the benefits of antibiotic treatment are considerably less. This developing resistance to antimicrobial therapy has been correlated with an exagerated inflammatory response of the open wound. The vessels within these wounds exhibit marked increase in vascular permeability. A protein rich fluid extravasates into the wound forming a fibrinous coagulum. As the coagulum accumulates, the contaminated wound becomes refractory to antibiotic treatment. It appears that the fibrinous coagulum surrounds the bacteria preventing them from contacting the topically or systemically administered antibiotics.

Hydrolysis of this surface coagulum by proteolytic enzymes considerably prolongs the effective period of antibiotic action. The efficacy of proteolytic enzymes as adjuncts to antibiotic treatment is directly related to its fibrinolytic activity. Topical treatment with an enzyme that has substantial fibrinolytic activity can enhance the wound's susceptibility to antibiotic treatment. This benefit of proteolytic enzymes must be weighed against any possible side effects. As a result of their fibrinolytic activity, the enzymes may interfere with wound healing or damage the host or local tissue defenses. The validity of these hypotheses are examined in these experimental studies.

MATERIALS AND METHODS

Enzyme

A Bacillus subtilus protease was the proteolytic enzyme employed in these experiments. This enzyme was supplied as a lyophilized powder by Flint Laboratories, (Norton Grove, Illinois). In previous studies, the protease exhibited considerable fibrinolytic activity and proved to be an effective adjunct to delayed antibiotic treatment of contaminated wounds. Before each experiment, the enzyme was solubilized in 0.9% sodium chloride to the desired concentration and filtered through a 0.22 u Millipore® filter to remove bacterial contaminants.

Standard Animal Model

Male, Hartley guinea pigs weighing 300-350 grams were anesthetized by an intraperitoneal injection of sodium pentobarbital. The backs of each animal was clipped, depilated with Surgex®, washed thoroughly with water, and swabbed with 70% alcohol. Two, standard, paravertebral incisions were made in each animal. The wounds measuring 3 cm in length extended down through the panniculus carnosus. Bleeding was stopped by sterile gauze pressure.

Wound Healing

The purpose of this first experiment was to examine the influence of topical enzyme treatment on wound healing. In 12 animals, 0.1 ml of the enzume (12,000 PC) was applied topically to one wound in each animal. The concentration of enzyme employed in these studies is sufficient to enhance the effectiveness of delayed antibiotic treatment.

The contralateral wound was subjected to a similar volume of 0.9% saline serving as the control. Ten minutes later all wounds were closed with microporous tape. Fourteen days post-wounding, the breaking strength of each wound was assessed. The breaking strength of a wound is a measure of the force required to disrupt a wound without regard to its dimensions.

This measurement was initiated by making two parallel incisions (3 cm in length) 6 mm from each wound. Each incision extended through the panniculus carnosus. Two clips were then attached to the divided skin edges adjacent to the wound at a distance of 3 mm from the healing wound. One clip remained stationary while the other clip was attached to a continuous drive motor with a screw gear advance by means of a strain gauge. This latter device was connected to a Hewlitt-Packard recorder through an amplifier system. As the drive motor pulled the clips at a rate of 8 mm/min., the strain gauge recorder system provided a measure of the applied tension (gram-force) necessary to disrupt the healing wound.

Resistance to Infection

The purpose of this phase of the study was to determine the effect of topical proteolytic enzyme treatment on the tissue's resistance to infection. In this experiment, 28 guinea pigs with standardized wounds were included in this study. One wound in each animal was subjected to a topical treatment with 0.1 ml of 0.9% saline serving as the control. Ten minutes later, each wound was blotted with a sterile gauze sponge. The animals were then subdivided into two treatment

groups. In one group, both wounds in each animal were contaminated by 3.3×10^5 Staphylococcus aureus in 0.1 ml of 0.9% saline. The wounds in the other group of animals were subjected to a larger bacterial inoculum (1.1×10^6). Five minutes after contamination, the edges of the wounds were approximated by tape.

On the fourth postoperative day, the inflammatory responses of the wounds were measured. The width of the indurated margin of each wound was recorded in mm. Each wound was opened and examined for evidence of purulent discharge. A relative estimate of the number of bacteria in each wound was made. The entire length of each wound was swabbed three times with a cotton-tipped applicator after which time the contaminated swab was immersed into 5 ml of 0.9% saline. The tube containing the bacterial suspension was then agitated on a Vortex® mixer for one minute. The number of bacteria in the suspension was then quantitated by standard serial dilution technics.

Leukocyte Phagocytosis and Intracellular Kill

Traumatic injury and subsequent bacterial contamination elicit an inflammatory response against infection. Alteration in the microvasculature occurs that allows extravasation of phagocytic cells into the tissue. These cells possess the capacity to ingest and kill invading bacteria. Once the microorganism is phagocytized, it is normally destroyed by intracellular digestion. Microbial death does not always follow phagocytosis, however, and certain bacteria will survive within the leukocyte for prolonged periods of time. This phase of the study

examined the effect of the proteolytic enzyme on the ability of the leukocyte to phagocytize and kill bacteria.

In vitro measurement of leukocyte function was patterned after the technic described by Mandel.[1] Fresh venous blood collected in heparinized tubes was combined with an equal volume of 3% Dextran and placed at a 45° angle for one hour in order to sediment the erythrocytes. The supernatant containing plasma, leukocytes, and platelets was centrifuged at 280 g for 12 minutes and the resulting button of white blood cells (10^5) was resuspended in 3.4 ml of Hank's balanced salt solution and 0.4 ml of autologous serum. The fresh autologous serum contains the opsonins and complement system that facilitates phagocytosis of foreign particles. To one half of the white cell suspensions was added 4 mg (12,000 PC units) of enzyme powder. The remaining cell suspensions were not subjected to the enzyme and served as controls. All tubes received 0.2 ml of a bacterial suspension (10^7) of S. aureus (ATCC 12,600). The tubes were then rotated at 12 rpm and maintained at 37° C.

Upon bacterial inoculation and at one and two hours post inoculation, a measured aliquot (0.1 ml) of fluid was removed from both test and control samples. This aliquot was diluted in sterile water (9.9 ml) and vortexed for 2 minutes in order to lyse the leukocytes. The number of bacteria within the water represents both the free bacteria as well as those phagocytized but viable within the cells. Knowledge of the total number of bacteria over time revealed the phagocytic bactericidal

capacity of the white cells.

A second aliquot (0.5 ml) was also removed upon inoculation and at one and two hours post inoculation and diluted in sterile saline (4.5 ml). Through differential centrifugation (280 g), the free bacteria (supernatant) were separated from the leukocytes (sediment). After sampling the sediment and supernatant, the leukocytes in the sediment were then lysed in sterile water in order to release any phagocytized yet viable bacteria. The number of free extracellular and intracellular bacteria was measured using standard serial dilution and plating technique. Quantitation of extracellular bacteria (supernatant) over time provides a measure of phagocytosis, while quantitation of intracellular bacteria (sediment) provides an index of the actual bactericidal action within the leukocyte.

RESULTS

Topical treatment of the wound with a proteolytic enzyme had no demonstrable effect on wound healing (Fig. 1). The breaking strength of the wound subjected to enzyme treatment did not differ significantly from that of the control wounds. Similarly, short term topical enzyme treatment had no damaging effect on the wound's resistance to infection. Wounds pretreated with enzymes were able to resist the development of infection to a degree similar to that of the control wounds pretreated with saline (Fig. 2). Enzyme treatment was associated with a significantly wider indurated wound margin than the controls. For the animals receiving 3.3×10^5 bacteria, an elevated bacterial count was associated with the widened indurated margins of

the enzyme treated wounds. For wounds receiving a higher level of inoculum, the bacterial counts of the saline and enzyme treated wounds were remarkably similar.

These inocuous effects of enzyme on the wounds *in vitro* are in sharp contrast to their deleterious influence on white blood cell function *in vivo*. In the presence of the enzyme, leukocytes were not able to phagocytize bacteria. As a result of this remarkable inhibition of bacterial ingestion, the leukocyte had no significantly bactericidal capicity. (Fig 3)

COMMENTS

In vivo, topical enzyme treatment for short periods of time appears to be inocuous. The presence of the enzyme in the wound did not interfere with either wound healing or wound resistance to infection. The only side effect encountered was a widened indurated margin which in one set of animals was correlated with an elevated bacterial count.

In vitro studies suggest that prolonged contact with the enzyme may have some potential deleterious effects. In the presence of the enzyme for one to two hours, the leukocyte's ability to phagocytize bacteria was not apparent. Consequently, enzyme treated leukocytes had no demonstrable capacity to kill bacteria. The period of time in which the leukocyte was exposed to theenzyme (*In vitro*) was substantially longer than the time of enzyme contact (10 minutes) with the wound surface. Limiting the period of enzyme wound treatment to this short time interval may reduce considerably its effect on white cell function *in vivo*.

The results of the study indicate that short term treatment of wounds with topical enzymes appears to be safe and is accompanied by few clinically significant side effects. The potential benefits of this treatment in experimental wounds as an adjunct to antibiotics considerably outweighs its damaging effects. Its clinical use in patients must await the results of carefully controlled clinical trials in which the efficacy of their treatment is evaluated.

1. Mandell, G.L.: Bactericidal action of aerobic and anaerobic polymorphonuclear neutrophils. Infect. Immunity 9: 337, 1974.

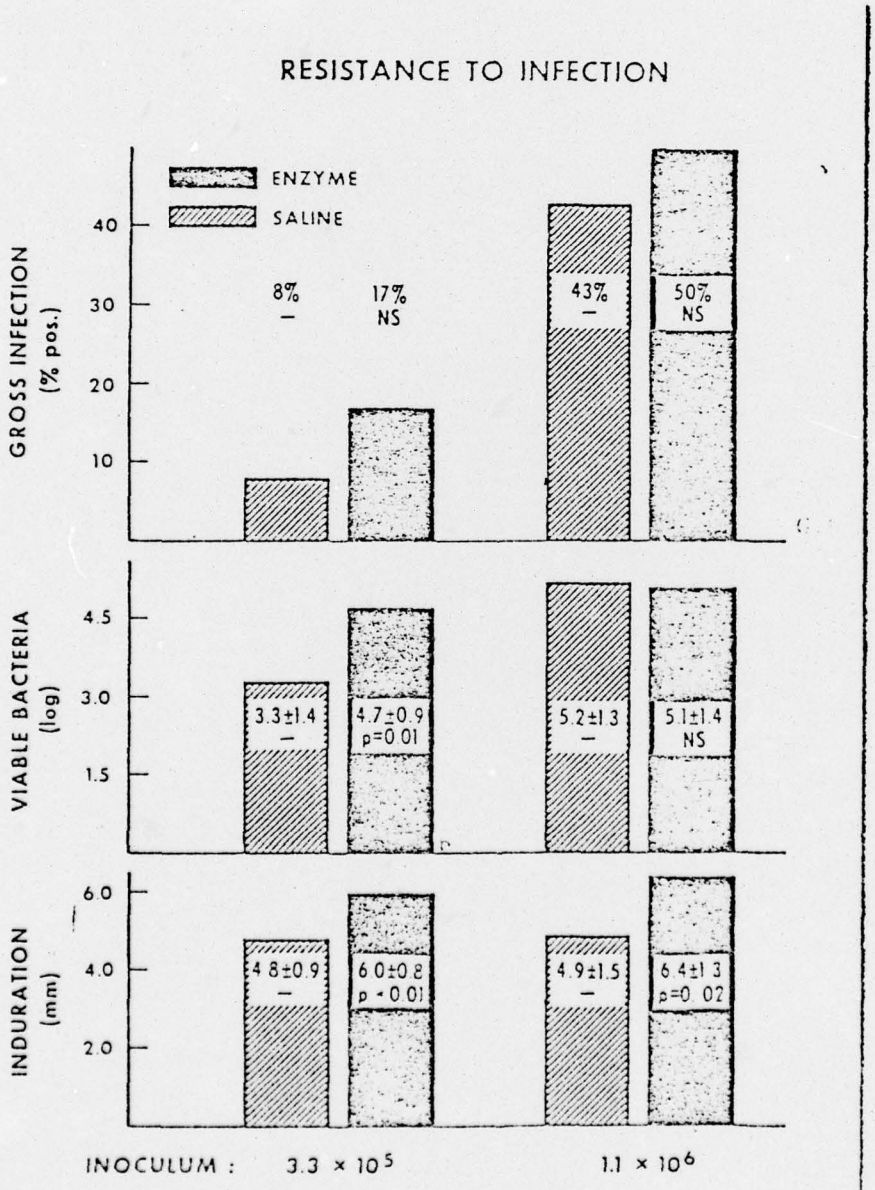
RESISTANCE TO INFECTION
ENZYME
SALINE
GROSS INFECTION (% pos.)
40
30
20
10
8%
—
17%
NS
43%
—
50%
NS
VIABLE BACTERIA (log)
4.5
3.0
1.5
3.3±1.4
—
4.7±0.9
p=0.01
5.2±1.3
—
5.1±1.4
NS
INDURATION (mm)
6.0
4.0
2.0
4.8±0.9
—
6.0±0.8
p < 0.01
4.9±1.5
—
6.4±1.3
p=0.02
INOCULUM : 3.3 × 10^5
1.1 × 10^6

Figure 2

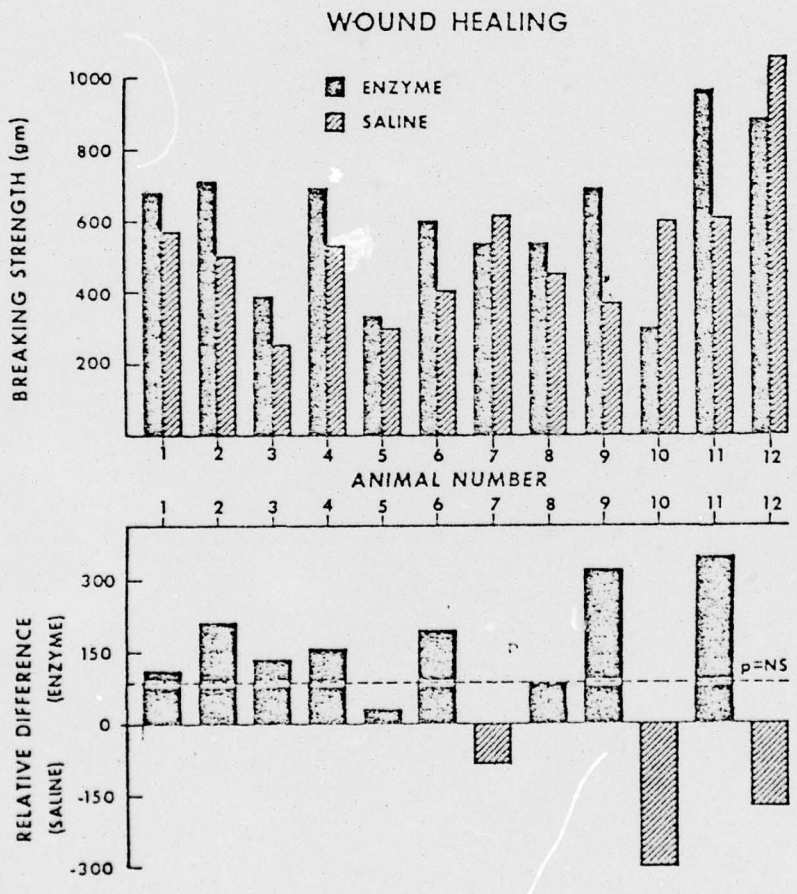
WOUND HEALING
ENZYME
SALINE
BREAKING STRENGTH (gm)
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11 12
ANIMAL NUMBER
1 2 3 4 5 6 7 8 9 10 11 12
RELATIVE DIFFERENCE (ENZYME) (SALINE)
300
150
0
-150
-300
p=NS

Figure 1

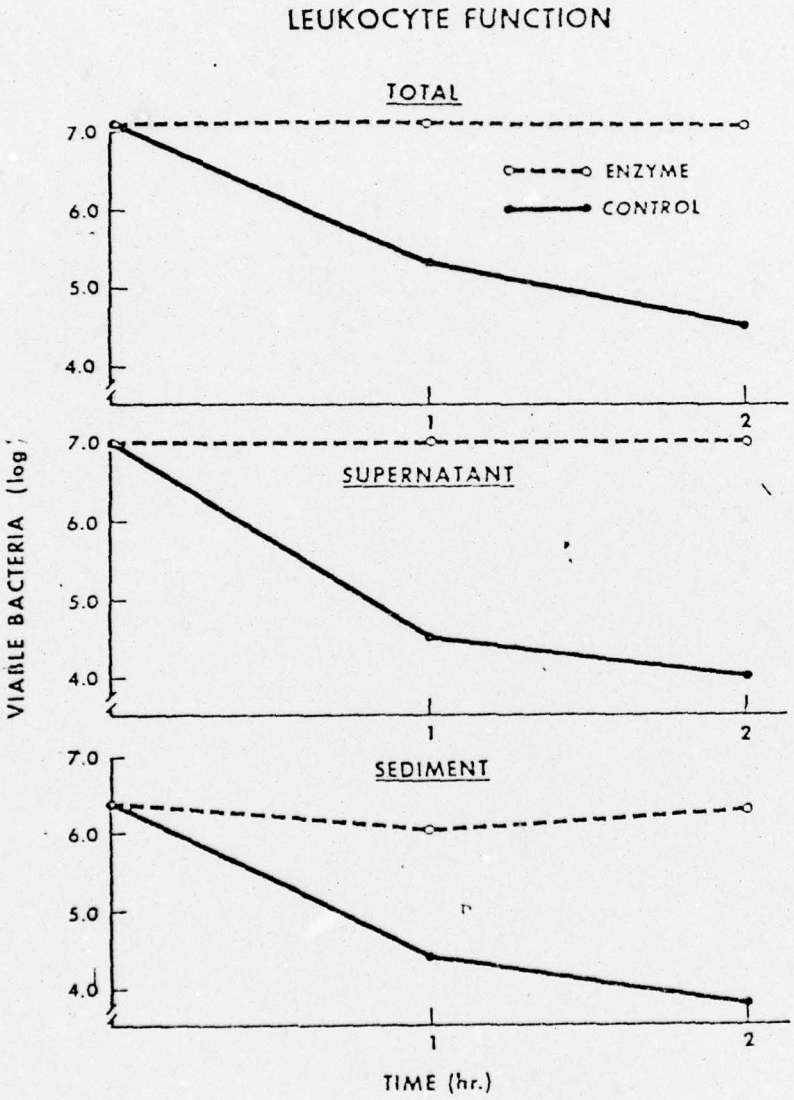
LEUKOCYTE FUNCTION
TOTAL
ENZYME
CONTROL
SUPERNATANT
SEDIMENT
VIABLE BACTERIA (log)
7.0
6.0
5.0
4.0
1
2
TIME (hr.)

Figure 3

IV. QUANTITATIVE ANAEROBIC MICROBIOLOGY OF BURN ESCHAR AND INFECTED WOUNDS

IV. QUANTITATIVE ANAEROBIC MICROBIOLOGY OF BURN ESCHAR AND INFECTED WOUNDS

A quantitative relationship exists between facultative bacterial species and the occurrence of burn wound sepsis and the incidence of wound infection. On this basis, quantitative bacteriology has been employed to predict the development of infection. The techniques employed to measure the number of wound pathogens have been standardized in our laboratory. The methodology involved includes weighing the tissue, homogenization of the clinical specimen, rapid slide measurement of the total number of bacteria in the homogenate followed by a viable count of the aerobic and facultative species. Frequently, the number of bacteria as ascertained by the rapid slide measurement differs considerably from the viable count. This discrepancy may be explained by the presence of non-viable organisms in the wound or obligate anaerobes in the specimen that succumb in the presence of oxygen. The validity of this hypothesis can be ascertained by standardized reliable and reproducible quantitative anaerobic microbiologic techniques. To this end, we have attempted to develope such technics. The reliability of a variety of proposed techniques is being confirmed by standard chemical and bacteriologic tests.

A. Chemical--During a quantitative anaerobic measurement, an anaerobic environment must be continually maintained. For each experiment, the environmental conditions are monitored by an indicator (resazurin) in the media and salt solution. When the redox potential is raised to a

level greater than -40 mv, the indicator exhibits a pink color indicating exposure to molecular oxygen. If the quantitative technic results in such a change, the measurement would probably discriminate against strict anaerobes and only allow growth of less oxygen-sensitive anaerobes (microaerophilic organisms).

B. Bacteriologic--Anaerobic bacteria are usually considered to be bacteria which grow only in the absence of oxygen. Yet, among the anaerobes it is apparent that degrees of sensitivity to molecular oxygen exist. Three obligate anaerobes with varying degrees of aerotolerance have been included in our bacteriologic evaluation. These include Clostridium hemolyticum (ATCC 9650 that exhibits maximum growth at pO_2 0.5%), Bacteriodes fragilis with maximum growth at pO_2 3%, and Fusobacterium nucleatum strain JCFan organism that grows best at pO_2 8%. A requirement for an accurate quantitative anaerobic technique is that it permit cultivation and measurement of these anaerobes in vitro. By recording changes in the numbers of these oxygen sensitive organisms as a result of the quantitative process, defects in the quantitative techniques have been identified.

Utilizing these standard techniques, the following technical problems have been encountered in the development of a standardized quantitative measurement for anaerobic bacteria.

C. Maintenance of Continuous Anaerobiosis

1. Gassing the Chamber with Carbon Dioxide

During the homogenization process, the clinical specimen is being

continually exposed to atmospheric oxygen. In an anaerobic quantitative bacterial analysis, the homogenization chamber must be purged of O_2. Simple exposure of the chamber to carbon dioxide prior to homogenization does not prevent exposure to oxygen. Similarly, gassing the chamber via a catheter placed above the homogenate is also inadequate. As a result of exposure to oxygen, death to the strict anaerobes has been encountered. Preliminary bacterial and chemical studies suggest that gassing the chamber via a catheter placed within the homogenate at the bottom of the chamber maintains an anaerobic environment. The influence of this anaerobic homogenization process on the survival of the anaerobes will be further tested during the coming year.

2. Pre-reduced Diluents

During homogenization of the clinical specimen and during serial dilution of the homogenate, a pre-reduced diluent is employed. Presently, we are employing a pre-reduced salt solution that is prepared in our laboratory and is relatively inexpensive. A pre-reduced solution containing chopped meat and various carbohydrates which is considerably more expensive than the salt solution may also be used. However, chopped meat diluent provides a more stable anaerobic environment than the pre-reduced salt solution. The influence of these solutions on the viability of anaerobes will be determined.

3. Pre-reduced Agar

The pre-reduced diluents are inoculated into pre-reduced agar media. Preliminary studies indicate that the concentration of agar

in the media determines the temperature at which the media gels. When the agar concentration is 2.5%, the gel point of the media is 46-48° C, a temperature at which most anaerobes will survive. When the agar concentration is raised to 3.5% the gel point of the media increases to 65-70° C, a temperature that results in death to the anaerobes. Further studies will be initiated to ensure that the gel point of the media will not influence viability of the anaerobes.

D. Temperature of Environment

When tissues are homogenized, considerable heat is given off, raising the temperature of the homogenate. This exothermic reaction has been minimized by placing the homogenization chamber in ice during our quantitation of facultative bacterial species and obligate aerobes. This precaution protects the organism from heat injury maintaining the viable count of the specimen. However, this chilling of the specimen may exert damaging effects on the anaerobic organism. Dowell points out that "specimens should not be refrigerated as chilling is detrimental to some anaerobes." The effect of this short period of hypothermia during homogenization on the viability of obligate anaerobes will be examined.

Following developing of this standard quantitative technique for anaerobic bacteria, it will be applied to experimental anaerobic infections of the burn and soft tissue wounds and then to clinical soft infections as well as burn wounds in humans.

PRACTICAL BACTERIOLOGIC MONITORING OF THE BURN WOUND*

Richard F. Edlich, M.D., Ph.D.*
George T. Rodeheaver, Ph.D.
Michael Spengler, B.S.
John Hiebert, M.D.
Milton T. Edgerton, M.D.

Dr. Edlich is a Junior Clinical Faculty Fellow of the American Cancer Society

Supported by a Grant from the United States Army Research and Development Command, Washington, D.C.

From the Department of Plastic Surgery, University of Virginia Burn Center, Charlottesville, Virginia 22901

INTRODUCTION

Burn wound sepsis remains one of the major causes of death of burnt children as well as adult burn victims.[20] This entity is characterized by progressive bacterial proliferation within the burnt tissue with subsequent invasion into adjacent viable tissue and eventually systemic dissemination.[24] Invasion of viable tissue by large numbers of bacteria, usually gram negative species, is associated with the clinical picture of abdominal distention, disorientation and shock. The onset of burn sepsis can often be predicted by the bacterial count of the burn tissue. When the quantitative bacterial count exceeds 100,000 (10^5) organisms per gram of tissue, the clinical consequences of sepsis are imminent. This quantitative relationship between the bacterial count and the development of burn wound sepsis is the fundamental basis for quantitative burn wound bacteriology.

Bacteriologic monitoring of the burn wound must take into account several parameters: type, number, location and antibiotic sensitivity of the bacteria. As a result of numerous technologic advances, these bacteriologic data can be made available to the burn surgeon in sufficient time to influence his therapeutic decisions. The microflora of the surface of the burn wound and the burn wound itself are examined separately by different sampling technics. (Table) For the surface microflora, the gauze capillarity technic is employed, while incisional biopsies are used to monitor the organisms in the burn wound. After sampling, suspensions of specimens are created which in turn are subjected to direct microscopic measurement, routine quantitative culture procedures and

immediate antibiotic sensitivity testing. Histologic examination of the burn wound is performed concomitantly with this bacteriologic examination to determine the depth of bacterial invasion as well as to detect mycotic or herpetic infections. It is the purpose of this report to review these methods and to point out their merits and shortcomings.

SURFACE BACTERIA

The surface of every burn wound is contaminated to some degree by bacteria.[9] Frequently, the level of surface bacteria does not reflect that of the underlying tissue. Similarly the organisms isolated from the surface may not be the same as the major subeschar pathogens accounting for burn wound sepsis. Despite these drawbacks, monitoring of surface counts is commonly employed in many burn centers.

Monitoring of surface bacterial counts can be accomplished by a variety of non-invasive methods.[1,2,4,7,10,11,23] In these technics the coefficient of friction of the two interacting surfaces, the burn wound and the sampling device, varies considerably. When the frictional forces between the surfaces are high, the surface of the burn wound is disrupted allowing access to bacteria in the outermost part of the burn wound. The bacterial recovery will reflect this penetration into the burn wound to include bacteria from the surface as well as within the tissues.

At our burn center, surface bacterial monitoring by the gauze capillarity technic[2] is employed prior to wound biopsy sampling. This procedure provides objective evidence of the number and type of surface bacteria, an index of the potential for subsequent bacterial invasion of the burn wound. Since this technic involves negligible frictional

forces between the sampling device and the burn wound, the bacteria recovered reflect surface rather than burn wound microbial colonization. This easily reproducible technic begins by neutralizing the antibacterial activity of antiseptic agents on the surface of the burn wound. Sterile gauze pads (2x2 in), 16 layers thick, are then placed in a sterile 100 mm petri dish. To this is added 4 to 5 ml of sterile solution which thoroughly soaks the gauze. Using aseptic technic, the gauze is then placed on the site to be cultured. The gauze is left in place for five minutes after which it is aseptically removed and rolled diagonally into a cylinder. The latter is placed into a sterile 125 ml Erlenmeyer flask containing 25 ml of 0.9% saline. Agitating the flask for two minutes, allows the bacteria adherent to the gauze to be suspended in solution. Aliquots (0.1 ml) from the original solution as well as serial dilutions are subjected to direct microscopic counts, quantitative culture procedures for aerobic bacteria and immediate antibiotic sensitivity testing.

WOUND BACTERIA

Biopsy Technique

The biopsy procedure is initiated by decontaminating the burn wound surface with a non-toxic antiseptic agent. Using aseptic technic, the surface of the wound is cleansed with an iodophor.[22] Five minutes later, the iodophor is removed and any residual agent inactivated by washing the wound surface with a solution of 0.9% sodium chloride containing sodium thiosulfate (1% w/v). The surface of the wound is then dried with a gauze sponge. Although others flame the biopsy to eliminate surface contaminants, this decontamination procedure should be done cautiously since heat may kill significant numbers of bacteria within the biopsy specimen.[10]

The biopsy is performed by excising a 2 cm x 1 cm sample of eschar which weighs approximately 0.5 gm (Fig. 1). The bacterial counts in tissue of this size or larger are sufficiently high to be readily susceptible to bacterial quantitation by the rapid slide technic.[14] When smaller specimens are subjected to rapid slide measurements, the total bacterial count susceptible to quantitation is considerably diminished limiting the reliability and accuracy of the technic. Incisional biopsies of the burn eschar only rarely produce bleeding that is not controlled by digital pressure. Local anesthesia is seldom required in this biopsy procedure. Biopsy samples for either aerobic bacterial quantitation or histologic examination are transported immediately to the laboratory in sterile glass tubes. Special anaerobic containers are employed for specimens that will be subjected to anaerobic bacterial isolation and culturing.

AEROBIC BACTERIAL QUANTITATION

The specimen is processed within 1 hour of its procurement. The biopsy is weighed and then suspended in a measured amount of 0.9% saline. The tissue may be macerated with a knife,[12,15] but a simpler approach is tissue homogenization using a sterile rotor knife blade. During homogenization, the tube containing the bacterial suspension should be immersed in a 4° C ice water bath.[9] This ice water mixture provides efficient cooling of the homogenate maintaining its temperature below a level that could result in hyperthermic injury to bacteria. The homogenate is then processed by direct microscopic examination, quantitative culture procedures for aerobic bacteria, and immediate antibiotic sensitivity

testing.

After making serial dilutions of the homogenate, aliquots of the diluted and undiluted solutions are inoculated on blood agar plates (BAP) and eosin-methylene blue (EMB) media containing an indicator dye, eosin-methylene blue mixture, which provides an indication of the number of Enterobacteriaceae in the specimen. The plates are incubated at 37° C for 24 hours before colony counts are performed. An appropriate plate with non-confluent growth containing between 30 and 300 colonies is selected for colony counts. The results are reported as bacterial count per gram of tissue.

Frequently, we supplement these bacteriologic studies with histologic examination of the burn tissue.[16,20] The tissue is examined for depth of microbial penetration, presence of an inflammatory response, evidence of microbial invasion of viable tissue and identification of blood vessel involvement. Using the histologic criteria described by Pruitt and Foley,[20] the diagnosis of invasive burn wound infection can then be made. Histologic examination of the burn wound is particularly valuable in the diagnosis of fungal infection. Mycotic infection of the burn wound is established solely by histologic criteria since, with the exception of candida, culture isolation of fungi has a low yield and is time consuming. The value of histologic examination of burn tissue is also of great value in the detection of herpetic lesions.[5]

ANAEROBIC BACTERIAL QUANTITATION

Whenever dead tissue is present, anaerobic infections may occur. Anaerobes have considerably different requirements for anaerobiosis.

Some grow on the surface of a solid media in the presence of air while others are unable to grow if the atmosphere contains as little as 0.3% oxygen or certain oxidized medium constituents. Considering this spectrum of aerotolerance, the methods employed for isolation of aerobes in burn wound specimens must allow recovery and quantitation of the least aerotolerant organisms.

Since many anaerobes can not tolerate even a very brief exposure to oxygen, the biopsy specimen should be obtained with minimum exposure to air. The tissue specimen should be transported immediately in an anaerobic tube or container.[6,26] Isolation and quantitation of the anaerobes is accomplished under anaerobic conditions in a glove box. During anaerobic homogenization, the sample is suspended in a measured amount of a pre-reduced salt solution which is also used for the serial dilutions.[8] Aliquots of the undiluted homogenate and its dilutions are then added to tubes of molten (50° C) pre-reduced brain heart infusion media containing 2.5% agar. Media containing more than 2.5% agar melts at higher temperatures which result in an irreversible hyperthermic injury to bacteria. The pre-reduced media as well as the diluent contain aqueous reazurin, an oxidation-reduction indicator of anaerobiosis. In its reduced form, it is colorless, an indication of an anaerobic environment. When the system is exposed to traces of oxygen, the reazurin is oxidized and imparts a distinct color to the media or solution.

The inoculated tubes are then placed in a spinner and rotated about their long axis. As the media cools during tube rotation, the bacteria are suspended in a thin film along the wall of the tube. After incubation at 37° C under anaerobic conditions for 24 to 48 hours, the colonies

are counted. Individual colony tupes are isolated, subcultured and incubated under aerobic, 10% CO_2 and anaerobic conditions to determine if the isolate is in fact anaerobic and not facultative. The colonies growing on plates incubated in 10% CO_2 and aerobic conditions are considered microaerophilic. At this point, the laboratory reports to the surgeon on the presence or absence of anaerobes or microaerophiles and indicates their morphology and gram staining characteristics. Identification and antibiotic sensitivity testing of the anaerobic isolates are determined subsequently.[8]

RAPID SLIDE TECHNIC

The rapid slide technic consists of a direct microscopic measurement of the total number of viable and dead bacteria in the suspension.[14] The major advantage of this technic is the speed with which the results are available to the surgeon, within 20 minutes after biopsy. In this measurement, a designated amount (0.01 ml) of the undiluted suspension, homogenate, and/or a serial dilution are spread uniformly over a dileneated 1 cm^2 area of a glass slide which then is placed on a warmer to dry the smear. (Fig. 2)

The smear is then subjected to the improved Gram stain technic[13] developed in our laboratory (Fig. 3). This technic provides a more reliable and accurate method of differentiating gram-negative from gram-positive organisms than the conventional Gram staining procedure. The major pitfall of the conventional technic is that gram-positive organisms are too easily decolorized by alcohol and judged to be gram-negative.

In the improved method, fixation of the bacteria is accomplished

by the addition of methanol onto the surface of the warm slide. Bacteria fixed by methanol are more resistant to decolorization than are bacteria fixed by drying or heat. When the methanol evaporates, the slide is then flooded with buffered crystal violet, which is allowed to stand for 60 seconds. The primary stain is then poured off the slide with the iodophor mordant. The slide is then flooded with more mordant that remains on the slide for one minute. The aqueous mordant I_2KI employed in the conventional technic is unstable and rapidly loses its iodine content during storage. The degree to which iodine is lost is increased by elevating the room temperature and repeated exposure to the environment. As the concentration of iodine in the mordant solution is reduced, bacterial smears become susceptible to decolorization. The problems of loss of iodine from the mordant can be remedied by employing an iodophor as the mordant. This iodine complex is stable and has a long shelf life.

The slide is then decolorized uniformly with 95% ethanol until the solvent flows colorlessly from the slide. Decolorization of the smear usually takes 5-10 seconds. Excess alcohol is removed by rinsing the slide with water. The counter stain, safranin, is then added to the slide for 60 seconds before it is washed off with water. Each slide is allowed to dry and is examined under oil immersion using a 100x objective. Ten separate fields of each smear are examined and the average number of bacteria per field are recorded. The average number per field is multiplied by the number of fields in the one cm^2 area (4000) giving the total number of bacteria in the 0.01 ml aliquot of the undiluted suspension. This number is multiplied by 50 if the original suspension volume was 5 ml

to give the total number of bacteria in the suspension (Fig. 3). When the number of bacteria in the smear of the undiluted suspension is too numerous to count, the number of bacteria per field in the first tenfold suspension dilution is checked. In these cases, the dilution factor of the homogenate is taken into account in the final calculations of the number of bacteria in the undiluted suspension. The shape, gram-staining characteristics and number of bacteria per sample size are reported in the final results (Fig. 4).

The rapid slide technic gives accurate and reliable measurement when 400 or more bacteria are in the 0.01 ml suspension delivered to the slide (2.5×10^5 organisms per gram of tissue). When less than this number of bacteria are added to the slide, bacteria are not detectable on microscopic examination.

The development of this technique does not replace quantitative serial dilution and plating technics. These latter technics are always performed concommitantly with the rapid slide technic since they allow speciation of the pathogen and antibiotic sensitivity testing.

IMMEDIATE ANTIBIOTIC SENSITIVITY TESTING DISKS

Rapid antibiotic sensitivity testing is performed under aerobic conditions directly on the bacterial suspension prepared from the clinical specimen rather than on single strains of bacteria isolated from the tissue.[25] Performing the antibiotic sensitivity test directly on the tissue sample allows the burn surgeon to receive the test results seven hours after receiving the specimen rather than 38-52 hours later, a time delay encountered with the conventional technic. Use of this

modification employed does not alter most of the standards recommended by the Food and Drug Administration since there is no inclusive change in the medium, agar depth or the antibiotic sensitivity disc.[3]

Aliquots of the bacterial suspensions prepared by the previous procedures are streaked in three directions onto the surface of Mueller-Hinton agar plates (5x150 mm) using sterile cotton swabs. After a three minute delay, antibiotic discs are applied to the surface of the agar with an automatic dispenser and pressed onto the surface with sterile forceps. After incubation at 37^{o} C for seven and for 18 hours, the zone of inhibition around each disc is measured with a ruler. The zone diameters as recommended by the Food and Drug Administration are used to interpret the susceptibility of the bacteria to the antibiotic.

In preliminary clinical and experimental studies, the changes in the test necessitated by using the clinical suspension did not alter significantly the interpretation of the antibiotic susceptibility. Even when larger numbers of bacteria were present in the suspension (10^{6}-10^{9}), variation in the inoculum size did not appreciably change the results of the antibiotic susceptibility tests. Reducing the standard Kirby-Bauer antibiotic susceptibility test to seven hours also did not limit the accuracy of the test.

As expected, the variable most difficult to standardize is the heterogenous inocula containing large numbers (10^{7}) of different organisms. A zone of inhibition interpreted as sensitive with one organism was occasionally masked by the presence of the confluent growth of another

organism whose zone of inhibition was considered resistant. We did not encounter the circumstance in which a number of sensitive species gave reactions interpreted as resistant when tested in combination. However, even in these cases it is possible that the results of mixed culture sensitivities may provide the most valid information in treating mixed infections since they most closely simulate the clinical situation. The merit of direct antibiotic sensitivity testing of clinical specimens must await further experimental and clinical studies in which the results of this proposed sensitivity test are shown to have some correspondence with the clinical response to treatment.

ANTIMICROBIAL CREAMS

For an antimicrobial cream to be effective against the organisms proliferating in the burn wound the agent must exhibit antibacterial activity against the major pathogen. Nathan[15] has recently devised a test which measures the susceptibility of the burn wound pathogens to commercially available antimicrobial creams. This antimicrobial sensitivity test is essentially a modification of the agar cup diffusion test for antibiotics. Measured amounts of the creams are delivered into the center of holes on an agar plate (standard brain heart infusion medium). The bacterial suspension is then added to melted agar (45° C) which is poured into the center of holes on an agar plate containing the antimicrobials to be tested. The contaminated fluid agar overlay solidifies within one minute. The test plates are inverted and incubated at 37° C for 6 to 24 hours.

This antimicrobial test provides an *in vitro* system to study the pharmacokinetics of a designated cream. Using this test, the influence

of the carrier on the activity of the cream can easily be appreciated. Some carriers limit the antibacterial activity of the antimicrobial agent while others permit maximal activity. (Fig. 5). The clinical value of this test to the burn surgeon in the selection of an appropriate antimicrobial cream for use against a specific pathogen is less certain. This uncertainty will be resolved when standards are developed similar to those for the disk sensitivity tests that can be used to interpret the susceptibility of the bacteria to the antimicrobial cream. However, it is important to point out that standardization of this cup-agar diffusion test may be more difficult than the disk test, particularly when clinical specimens are employed. The inoculum size and the length of incubation of clinical specimens significantly influence the interpretation of the results of the agar-cup diffusion test, a circumstance rarely encountered with the disk test (Fig. 3A and B).

INDIRECT MEASURES OF THE BACTERIAL COUNT

Several indirect measures of the wound bacterial count are available to the burn surgeon. Examination of burn wounds with a Wood's lamp allows the clinician to identify early pseudomonas infection.[19] A fluorescent pigment is elaborated by many pathogenic strains of Pseudomonas aeroginosa and is detectable under long-wave ultraviolet light. The point at which fluorescence is seen upon the wounds of the burn patient in a partially darkened room is approximately 1,000 bacteria/ $cm.^2$ It is indeed fortuitous that this concentration is less than the minimum density at which invesive burn wound sepsis occurs. This immediate and early documentation of pseudomonas colonization well before clinical signs of infection facilitates timely debridement that may be lifesaving

in some patients.

The "take" of skin grafts on the surface of granulating burn wounds is another rather reliable indication of the wound bacterial count.[21] When the bacterial population is 10^5 or fewer organisms per gram of tissue, autografting with split thickness skin is almost uniformly successful. Skin homograft also successfully "takes" only when the colony count was 10^5 or less per gram in the tissue biopsy.

SUMMARY

A comprehensive picture of the burn wound microflora is now possible as a result of recent technologic advances. The microflora of the burn wound can be characterized with respect to its number, type, location and antibiotic sensitivity. These parameters can be measured in sufficient time to influence the decision of the burn surgeon. The microflora of the surface of the burn wound and the burn wound itself are examined separately by different sampling technics. For the surface microflora, the gauze capillary technic is employed, while incisional biopsies are used to monitor the organisms in the burn wound. After sampling, suspensions of the specimens are created which in turn are subjected to direct microscopic measurement, quantitative culture procedures and immediate antibiotic sensitivity testing. Histologic examination of the burn wound is performed concommitantly with this bacteriologic examination to determine the depth of bacterial invasion as well as to detect the presence of either mycotic or herpetic infections.

LEGENDS FOR ILLUSTRATIONS

Figure 1. The incisional biopsy is performed by excising a 2 cm x 1 cm sample of eschar (A,B). Biopsy specimens for either aerobic bacterial quantitation or histologic examination are transported immediately to the laboratory in sterile glass tubes. Special anaerobic containers are employed for specimens that will be subjected to anaerobic bacterial isolation and culturing.

Figure 2. Calculation of the results of the rapid slide measurement.

Figure 3. Improved Gram stain technic.

Figure 4. Morphologic and gram staining characteristics of bacteria.

Figure 5. The carriers in the antimicrobial creams exert considerable influence on their antibacterial activity.

Figure 6. The inoculum size (A) and the incubation time (B) have considerable influence on the interpretation of the results of the sensitivity tests with antimicrobial creams.

(TABLE)

Sampling Technic	Direct Microscopic Count	Quantitation		Antibitoic Sensitivity		Histology
		Aerobic	Anaerobic	Disc	Cream	
Gauze Capillarity	+	+	-	+	+	-
Wound Biopsy	+	+	+	+	+	+

REFERENCES

1. Brentano L and Gravens DL: A method for the quantitation of bacteria In burn wounds. Appl Microbiol 15: 670, 1967.

2. Clarkson JG, Ward CG, and Polk HC: Quantitative bacteriologic study of the burn wound surface. Surg Forum 18: 506, 1967.

3. Department of Health, Education and Welfare. Food and Drug Administration: Proposed rule making. Fed. Regis. 36: 6899, 1970.

4. Eade GG: The relationship between granulation tissue, bacteria, and skin grafts in burned patients. Plast Reconst Surg 22: 42, 1958.

5. Foley FD, Greenawald KA, Nash G and Pruitt BA Jr: Herpesvirus infection in burned patients. New Eng J Med 282: 652, 1970.

6. Fulghum RS. Mobile anaerobe laboratory. Appl Microbiol 21: 769, 1971.

7. Georgiade NM, Lucas M, Georgiade R, and Garrett W: The use of a new potent topical antibacterial agent for the control of infection in the burn wound. Plast Reconst Surg 39: 349, 1967.

8. Holdeman LV and Moore WEC: Anaerobic bacteriology manual. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, 1975.

9. Lawrence JC and Lilly HA: A quantitative method for investigating the bacteriology of skin: its application to burns. Brit J Exp Path 53: 550, 1972.

10. Levine NS, Lindberg RB, Mason AD, and Pruitt BA: The quantitative swab culture and smear: A quick simple method for determining the number of viable aerobic bacteria on open wounds. J Trauma 16: 89, 1976.

11. Levine NS, Lindberg RB, Salisburg RE: A comparison of coarse-mesh gauze versus biologic dressings on granulating wounds. Amer. J. Surg. 131: 727, 197

12. Loebl EC, Marvin JA, Heck EL, Curreri PW, and Baxter CR. The use of quantitative biopsy cultures in bacteriologic monitoring of burn patients. J Surg Res 16: 1, 1974.

13. Magee CM, Rodeheaver G, Edgerton MT, and Edlich RF: A more reliable gram staining technic for diagnosis of surgical infections. Amer J Surg 130: 34, 1975.

14. Magee CM, Haury BB, Rodeheaver GT, Edgerton MT, and Edlich RF: A rapid technique for quantitating wound bacterial count. Amer J Surg (In Press).

15. Marvin JA, Heck CL, Loebl EC, Curreri PW, and Baxter CR: Usefulness of blood cultures in confirming septic complications in burn patients: Evaluation of a new culture method. J Trauma 15: 657, 1975.

16. McKeel DW Jr., Goodwin MN Jr, Worley BL and Lindburg RB: Definitive identification of burn wound fungi in fatally burned military personnel by culture and by histopathology, United States Army Institute of Surgical Research Annual Research Progress Report, June 30, 1972, BAMC, Fort Sam Houston, Texas Sec. 57.

17. Nash G, Foley FD, Goodwin MN, Jr., Bruck HM, Greenawald KA, and Pruitt BA, Jr. Fungal burn wound infection. JAMA 215: 1664, 1971.

18. Nathan P. Personal Communication.

19. Polk HC Jr, Ward CG, Clarkson JG, and Taplin D: Early detection of Pseudomonas burn infection. Clinical experience with Wood's light fluorescence. Arch Surg 98: 292, 1969.

20. Pruitt BA, and Foley Fd: The use of biopsies in burn patient care. Surgery 73: 887, 1973.

21. Robson MC and Krizek TJ: Predicting skin graft survival. J Trauma 13: 213, 1973.

22. Rodeheaver GT, Turnbull V, Edgerton MT, Kurtz L, and Edlich RF: Pharmacokinetics of a new skin wound cleanser. Amer J Surg 132: 67, 1976.

23. Saymen DG, Nathan P, Holder IA, and MacMillan BG. Infected surface wounds: an experimental model and a method for the quantitation of bacteria in infected tissues. Appl Microbiol 23: 509, 1972.

24. Teplitz C, Davis D, Mason AD Jr., and Moncrief JA: Pseudomonas burn wound sepsis. I. Pathogenesis of experimental Pseudomonas burn wound sepsis. J Surg Res 4: 217, 1964.

25. Verklin R, Rodeheaver G, Hueston R, Edgerton MT, and Edlich RF: Rapid antibiotic disc sensitivities of burn eschar and infected wounds. Surg. Gyn. Obst. In Press.

26. Wilkins TD, Jimenez-Ulate F: Anaerobic specimen transport device. J. Clin. Micro 2: 441, 1975.